Original Article

Resveratrol Partially Suppresses Inflammatory Events but Does not Affect Stroke Onset in Stroke-Prone Spontaneously Hypertensive Rats

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Aim: Resveratrol has been shown to mimic the beneficial effects of dietary restriction (DR). We previously reported that DR delays stroke onset and extends the lifespan in Stroke-Prone Spontaneously Hypertensive rats (SHRSP). Therefore, we examined whether resveratrol mimics DR and delays stroke onset in SHRSP.

Methods: Cerebrovascular endothelial cells (CVECs) from SHRSP were treated with resveratrol, and the inflammatory gene expression levels and NF κ B protein levels were measured. In order to address the effects of resveratrol *in vivo*, SHRSP (male, 10 weeks of age) were fed an experimental diet containing several doses of resveratrol (0 - 0.05% (w/w)), after which we measured the plasma cytokine levels and examined the stroke onset and lifespan.

Results: Treatment with resveratrol (100 μ M, 24 hours) in CVECs from SHRSP significantly decreased the interleukin (IL)-1 β -induced monocyte chemoattractant protein-1 (MCP-1) mRNA expression levels and p50 and p65 protein levels in the nuclear fraction. When the SHRSP were fed a diet containing resveratrol for one week, the resveratrol treatment did not affect the plasma lipid and glucose levels, body weight or weight of each tissue. Resveratrol slightly, but not significantly, decreased the plasma levels of IL-1 β and MCP-1 compared with that observed in the control group. In addition, resveratrol decreased the IL-1 β and MCP-1 mRNA expression levels in the brain versus the control animals. However, no doses of resveratrol delayed stroke onset or extended the lifespan in SHRSP.

Conclusions: In this study, resveratrol did not delay stroke onset in SHRSP, although it partially suppressed systemic and cerebral inflammation. These results suggest that resveratrol does not mimic the beneficial effects of DR on stroke *in vivo*.

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Key words: Resveratrol, Inflammation, Cerebrovascular endothelial cells, Stroke, SHRSP

Introduction

Resveratrol (trans-3,4',5-trihydroxystilbene), a phytoestrogen found in grapes, cranberries, peanuts and other plants, has become a popular ingredient in dietary supplements, as it has been shown to exert

anti-aging effects in various animal models¹⁾. The effects of resveratrol are similar to those of dietary restriction (DR) via sirtuin (SIRT)1 activation-dependent and -independent pathways²⁾ and include improvements in insulin sensitivity, endurance and overall survival in obese mice. Baur *et al.* demonstrated that mice fed a high-calorie diet with resveratrol express increased insulin sensitivity, reduced fat deposition in the liver, a greater number of mitochondria per cell and an extended lifespan compared to mice fed a high-calorie diet, without influencing body weight³⁾. Lagouge *et al.* also reported that mice fed a

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high-calorie diet with resveratrol exhibit a similar phenotype and show significant suppression of body weight gain⁴). On the other hand, it has been reported that resveratrol fails to extend the lifespan in mice fed a standard diet, although it mimics the beneficial effects of DR⁵). These findings suggest that the beneficial effects of resveratrol may be limited to certain disease conditions.

Previous studies have investigated the effects of resveratrol in ischemic model animals. Resveratrol attenuates ischemic brain damage by increasing the expression of matrix metalloproteinase-2 and vascular endothelial growth factor⁶) and exerting anti-oxidative, anti-inflammatory and anti-apoptotic effects^{7, 8}). There is one report on the effects of resveratrol in Stroke-Prone Spontaneously Hypertensive rats (SHRSP), which showed the protective effect of resveratrol against oxidative damage and glycoxidative stress only, not on stroke onset⁹). In addition, there are two reports of the protective effects of resveratrol against oxidative DNA damage in smooth muscle cells from SHRSP^{10, 11}). However, cell damage to cerebrovascular endothelial cells (CVECs) appears to be more important than that induced in smooth muscle cells with regard to stroke onset. Nevertheless, it remains unclear whether resveratrol protects CVECs from inflammatory stress and suppresses the incidence of spontaneous stroke.

We previously reported that inflammation, especially IL-1 β signaling, is important for stroke onset¹²) and that DR suppresses both systemic and local inflammation and markedly delays stroke onset in SHRSP¹³). If resveratrol mimics the beneficial effects of DR, it may also delay stroke onset and extend the lifespan of SHRSP. In order to clarify this hypothesis, we examined whether resveratrol effectively delays stroke onset in SHRSP.

Methods

Cerebrovascular Endothelial Cells

SHRSP (male, 4 weeks of age) were killed, the brains were removed and CVECs were harvested, as previously described¹³). The CVECs were cultured with Endothelial Cell Basal Medium-2 (Lonza, Walkersville, MD) and used within 10 passage numbers. The CVECs were subsequently plated onto a collagen-coated 60-mm dish and cultured until confluent with Endothelial Cell Basal Medium-2 (5% FCS) in order to examine the effects of resveratrol on the cells. The cells were then growth arrested in serum-free medium for 24 hours and incubated with 0, 1, 10 or 100 μ M of resveratrol (Sigma-Aldrich Inc., St Louis, MO)

under 10 ng/mL rat recombinant IL-1 β (Sigma-Aldrich Inc.) stimuli in serum-free medium for 24 hours. Total RNA and protein were extracted after the incubation period, following which the mRNA expression levels were measured using quantitative RT-PCR and the protein levels were assessed using a Western blot analysis.

Quantitative RT-PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen Co., Carlsbad, CA) and reverse transcribed using an Omniscript RT Kit (QIAGEN, GmbH, Germany) and oligo (dT) primers. The reactions were performed using the Thermal Cycler Dice Real Time System (Takara Bio Inc., Shiga, Japan), and the results are expressed as the copy number ratio of the target mRNA to GAPDH mRNA. The following rat-specific primer pairs were used:

IL-1 β forward 5'-AGCAGCTTTCGACAGT-GAGGAGAA-3';

reverse 5'-TCTCCACAGCCACAATGAGT-GACA-3';

MCP-1 forward 5'-TGCTGTCTCAGCCA-GATGCAGTTA-3';

reverse 5'-TACAGCTTCTTTGGGACACCT-GCT-3';

ICAM-1 forward 5'-TGCAGGTGAACT-GCTCTTCCTCTT-3';

reverse 5'-AGCTTCCAGTTGTGTCCACTC-GAT-3';

eNOS forward 5'-ATGCCTACAGCATTG-GTTGCAAGG-3';

reverse 5'-AGCATATGAAGAGGGCAGCAG-GAT-3';

GAPDH forward 5'-TGATGCTGGTGCT-GAGTATGTCGT-3';

reverse 5'-TCTCGTGGTTTCACACCCATCAA-3'.

Western Blot Analysis

Nuclear (for NF κ B subtypes) or whole (for the AMPK signaling pathway) protein from CVECs was extracted with the Nuclear Extract Kit (Active Motif, Inc., Carlsbad, CA) or PRO-PREP (iNtRON Biotechnology, Inc., Gyeonggi-do, Korea) in accordance with the manufacturer's instructions. The protein concentrations were measured with Dc Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Then, protein (10 μ g) separated via SDS-PAGE (12% gel) was transferred onto an Immun-Blot PVDF Membrane (Bio-Rad Laboratories) and immunoblotted with specific primary antibodies: NF κ B p50 (sc-7178), p52 (sc-298), p65 (sc-372), Rel-B (sc-226),

c-Rel (sc-71), SIRT-1 (sc-74465) and β -actin (sc-47778) (1:200 - 1000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), AMP-activated protein kinase α (AMPK α) (2603), phospho-AMPK α (Thr172) (2535), AMPK β (4150), phospho-AMPK β (Ser108) (4181), acetyl-CoA carboxylase (ACC) (3676) and phospho-ACC (Ser79) (3661) (1:1000 dilution, Cell Signal Technology, Inc., Boston, MA). Peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) (1:2000 dilution) was used as the secondary antibody. Bands were visualized using an enhanced chemiluminescence system (GE Healthcare), and the protein expression levels were adjusted by the β -actin protein level in each sample.

Animals

Male SHRSP/Izm were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained under specific pathogen-free conditions in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) with a 12-hour light/dark cycle. The animals had free access to the normal laboratory diet (CE-2; CLEA Japan Inc., Tokyo, Japan) and water until 10 weeks of age, at which time the experiments were performed. Experimental diets (caloric composition: 20% protein, 10% fat and 70% carbohydrate; #D07042709) containing several doses of resveratrol (0 - 0.05% (w/w)) were purchased from Research Diet Inc. (New Brunswick, NJ). All rats were given drinking water supplemented with 1% NaCl at the beginning of the experimental period. Each group consisted of five (for plasma and brain biochemistry) or eight (for stroke onset) rats, each of which was kept in a separate steel cage and killed after one week or monitored for neurological symptoms and physiological changes to detect the onset of stroke. All animal experiments were conducted with the approval of the National Institute of Health and Nutrition Ethics Committee on Animal Research.

Food Intake, Body Weight and Blood Pressure

Food intake, body weight and blood pressure were measured as described in our previous study¹⁴.

Plasma Chemistry

The plasma IL-1 β and soluble intercellular adhesion molecule-1 (sICAM-1) levels were determined with Quantikine rat IL-1 β or sICAM-1 Immunoassay Kits (R&D Systems, Inc., Minneapolis, MN), respectively. The plasma monocyte chemoattractant protein-1 (MCP-1) levels were determined using an Endogen Rat MCP-1 ELISA Kit (Pierce Biotechnology, Inc., Rockford, IL). The plasma NO $_x$ (Nitrate/

Nitrite) levels were determined using a Parameter Total Nitric Oxide and Nitrate/Nitrite kit (R&D Systems, Inc.).

Measurement of the Plasma Resveratrol Levels

The plasma resveratrol levels were measured with the HPLC method, as previously described¹⁵⁻¹⁷. Plasma samples were pretreated with β -glucuronidase and sulfatase solution to digest the conjugated form. Resveratrol was then extracted twice with 1 mL of ethyl acetate, after which the organic layer was transferred to a glass tube, mixed with 10 μL of 0.2% ascorbic acid and dried under a nitrogen stream. The residue was then reconstituted with 200 μL of 3% acetic acid:acetonitrile (80:20, v/v), and applied to the HPLC system described below. The HPLC system was a Shimadzu HPLC-VP system (Shimadzu Corporation, Kyoto, Japan) with an L-column ODS (Chemical Inspection & Testing Institute, Tokyo, Japan) (4.6×250 mm, 5 μm particle), UV detector (at 306 nm) and column oven (40°C). Resveratrol was eluted with a gradient of 3% acetic acid (phase A) and mixture of 3% acetic acid:acetonitrile (20:80, v/v) (phase B). The gradient protocol was as follows: 0-2 minutes, 22% phase B; 2-6 minutes, linear gradient from 22 to 30%; 6-14 minutes, linear gradient from 30 to 50% B; 14-18 minutes, increasing to 60% B; 18-25 minutes, linear gradient from 60 to 100% B; 25-35 minutes, 100% phase B. Under chromatographic conditions, *trans*-resveratrol and the internal standard were eluted at 15 and 20 minutes, respectively. The peak height ratio method (*trans*-resveratrol/internal standard) was used for quantitation.

Gene Expression in the Brain

The rats were killed one week after the initiation of the experimental diet, and the brains (cerebrum) were removed for total RNA extraction. Total RNA was extracted with the TRIzol Plus RNA Purification System (Ambion, Austin, TX) and converted to cDNA for quantitative RT-PCR.

Stroke Onset

The onset of stroke was assessed according to the appearance of neurological symptoms (hyperkinetic/hypokinetic behaviors, hyper/hyposensitivity [manipulation], proleg lift or piloerection) and physiological changes (a decrease in body weight or food intake or increase in drinking water intake) as previously described¹⁴. Two researchers checked for these symptoms at 10:00 am every day.

Fig. 1. Effects of resveratrol on the expression of inflammatory genes in the CVECs

SHRSP CVECs were treated with resveratrol (0 - 100 μ M) under 10 ng/mL of IL-1 β -stimuli for 24 hours. Total RNA was extracted to determine the mRNA expression levels using quantitative RT-PCR. The bars show the SEM, * P < 0.05 and *** P < 0.001 vs. 0 μ M Res in IL-1 β -stimuli according to the one-way ANOVA with a Bonferroni post-hoc test. n = 3.

Histological Analysis

The brain was fixed in 10% buffered formalin solution, sliced into five coronal sections, embedded in paraffin, cut into 4- μ m-thick sections and stained with hematoxylin-eosin. Sites of hemorrhage were defined as areas with clustering blood cells. Regions of infarction were defined as areas with necrosis, gliosis and glial scarring. Lesions of infarction, hemorrhage or edema were classified into five degrees based on the percentage of the lesion area to the whole area in each section, as follows: (-), no abnormalities; (+), 0-10%; (++) , 10-20%; (+++) , 20-30%; (++++), 30-40%; (+++++) , more than 40%.

Statistical Analysis

The data are presented as the mean $\pm$ SEM. Comparisons of data from multiple groups were performed using a one-way ANOVA with a Bonferroni post-hoc test (SPSS 18.0J for Windows, IBM Co. Armonk, NY). Kaplan-Meier curves were obtained for the onset of stroke and survival, and the groups were compared using the log-rank test (Statview 5.0, Aba-

cus Concepts, Piscataway, NJ). A P value of <0.05 was considered to be statistically significant.

Results

Resveratrol Partially Suppressed Inflammatory Events in the SHRSP CVECs

SHRSP have higher plasma IL-1 β levels than SHR, which might be a cause of stroke onset in SHRSP models¹². Prior to the *in vivo* study, we confirmed the anti-inflammatory effects of resveratrol on CVECs *in vitro*. A high concentration (100 μ M) of resveratrol significantly suppressed the IL-1 β -induced MCP-1 mRNA expression in the CVECs (Fig. 1). In addition, a high concentration of resveratrol also significantly suppressed the p50 and p65 protein levels in the nuclear fraction (Fig. 2). On the other hand, a high concentration of resveratrol significantly increased the IL-1 β mRNA expression and slightly, but not significantly, increased the Rel-B and c-Rel protein levels in the nuclear fraction. Resveratrol did not affect the expression of IL-1 β -induced ICAM-1

Fig. 2. Effects of resveratrol on the expression of NF κ B proteins in the CVECs

SHRSP CVECs were treated with resveratrol (0 - 100 μ M) under 10 ng/mL IL-1 β -stimuli for 24 hours. Nuclear protein was extracted to determine the NF κ B protein levels using a Western blot analysis. The bars show the SEM, * P < 0.05 vs. 0 μ M Res in IL-1 β -stimuli according to the one-way ANOVA with a Bonferroni post-hoc test. The data are presented as the mean $\pm$ SEM of densitometric ratios (non-stimuli was set at 100). n = 3.

Fig. 3. Effects of resveratrol on the AMPK signals in the CVEC

SHRSP CVEC were treated with resveratrol (100 μM) with or without IL-1β (10 ng/mL) for 24 hours. After the incubation period, whole cell proteins were extracted. The AMPK signal protein levels were determined using a Western blot analysis. The data are presented as the mean ± SEM of densitometric ratios (non-stimuli was set at 100). $n=3$.

Table 1. Plasma levels of lipids, glucose and resveratrol

	Resveratrol (w/w)					P value
	Control	0.001%	0.005%	0.01%	0.05%	
TC (mg/dL)	123.7 ± 2.0	119.5 ± 2.9	122.3 ± 3.3	117.5 ± 3.9	112.5 ± 5.2	0.247
TG (mg/dL)	213.8 ± 27.1	165.9 ± 14.7	188.9 ± 33.5	169.2 ± 32.7	152.5 ± 29.0	0.591
Glucose (mg/dL)	176.3 ± 4.0	191.0 ± 9.2	174.1 ± 6.6	176.3 ± 6.5	184.2 ± 7.6	0.422
Resveratrol (ng/mL)	n.d.	10.5 ± 0.2	23.2 ± 3.3	26.0 ± 7.3	64.1 ± 12.4	0.001

The data are presented as the mean ± SEM. Plasma samples were prepared one week after the initiation of the experimental diet. The plasma levels of TC (total cholesterol), TG (triglycerides) and glucose were measured using enzymatic methods. The plasma resveratrol levels were measured using the HPLC method. The *P* values of a one-way ANOVA are shown. n.d.; non-detectable. *n* = 5 in each group.

Table 2. Body weight and weights of each tissue

	Resveratrol (w/w)					P value
	Control	0.001%	0.005%	0.01%	0.05%	
BW (g)	271.2 ± 4.4	270.2 ± 6.2	272.1 ± 4.1	272.2 ± 4.1	276.9 ± 4.8	0.880
Liver (g)	11.06 ± 0.47	10.77 ± 0.22	10.63 ± 0.31	10.51 ± 0.27	10.38 ± 0.36	0.611
Spleen (g)	0.53 ± 0.01	0.52 ± 0.02	0.53 ± 0.02	0.54 ± 0.01	0.55 ± 0.02	0.697
Vis Fat (g)	4.05 ± 0.14	4.18 ± 0.20	3.88 ± 0.15	3.92 ± 0.24	4.19 ± 0.25	0.703
Brain (g)	1.68 ± 0.02	1.65 ± 0.02	1.67 ± 0.02	1.66 ± 0.01	1.66 ± 0.02	0.812

The data are presented as the mean ± SEM. The body weight (BW) and liver, spleen, visceral fat (epididymal fat) and brain (cerebrum) weights were measured one week after the initiation of the experimental diet. The *P* values of a one-way ANOVA are shown. *n* = 5 in each group.

or IL-1 β -suppressed eNOS mRNA. These results suggest that resveratrol partially suppressed inflammation in the CVEVs *in vitro*.

Resveratrol Activated the AMPK Signaling Pathway in the SHRSP CVECs

We also confirmed the activation of the AMPK signaling pathway by resveratrol in the CVECs (Fig. 3). IL-1 β increased the AMPK α , AMPK β and ACC protein levels, but not the phosphorylation of these proteins, in the CVECs. Resveratrol decreased both the AMPK α and AMPK β protein levels, whereas it slightly increased the phosphorylated AMPK α and AMPK β levels. Furthermore, resveratrol decreased the total ACC level, the target protein for AMPK, and increased phosphorylated ACC. These results indicate that resveratrol activated AMPK signals in CVEVs, similar to that observed in other cells. However, why the total AMPK and ACC levels were decreased by resveratrol treatment remains unclear.

Resveratrol Slightly Suppressed Systemic and Local Inflammation in SHRSP

We previously reported that 50% DR signifi-

cantly suppresses systemic inflammation (plasma levels of IL-1 β and MCP-1) and delays the onset of stroke in SHRSP¹³). In the current study, the SHRSP were fed a control diet or diets containing various doses (0.001 - 0.05%) of resveratrol to determine the anti-inflammatory effects of resveratrol *in vivo*. The plasma levels of resveratrol increased in a dose-dependent manner (Table 1). However, no doses of resveratrol for the one-week feeding protocol affected the plasma levels of lipids and glucose, body weight or the weight of each tissue in the SHRSP (Table 1 & 2). Resveratrol slightly suppressed the plasma levels of IL-1 β and MCP-1 (Fig. 4A). Moreover, 0.001% resveratrol significantly suppressed the sICAM-1 levels (Fig. 4A), while a higher dose did not. In addition to the plasma levels of inflammatory cytokines, the IL-1 β and MCP-1 mRNA expression levels in the brain were decreased by resveratrol (Fig. 4B). In particular, the eNOS mRNA expression levels were increased by resveratrol in the brain, although the plasma levels of NOx were decreased. On the other hand, 0.05% resveratrol significantly increased the ICAM-1 mRNA expression levels in the brain. These results suggest that resveratrol slightly suppressed systemic and local

A Plasma

B mRNA in the brain

Fig. 4. Effects of resveratrol on inflammatory events *in vivo*

The SHRSP were killed, and (A) the plasma levels of inflammatory cytokines and (B) gene expression levels of inflammatory cytokines in the brain (cerebrum) were measured one week after the initiation of the experimental diets containing various doses of resveratrol (0 - 0.05% (w/w)). The bars show the SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control according to the one-way ANOVA with a Bonferroni post-hoc test. $n = 5$ in each group.

Table 3. Body weight, blood pressure and food intake

	Resveratrol (w/w)					<i>P</i> value
	Control	0.001%	0.005%	0.01%	0.05%	
BW (g)	266.8 ± 2.8	263.5 ± 3.9	263.1 ± 3.3	262.7 ± 4.6	262.6 ± 4.6	0.934
SBP (mmHg)	194 ± 4	204 ± 3	200 ± 3	199 ± 3	201 ± 3	0.344
Intake (g/rat/day)	24.3 ± 0.2	23.8 ± 0.3	24.3 ± 0.5	24.8 ± 0.4	25.0 ± 0.3	0.152

The data are presented as the mean ± SEM. The body weight (BW) and systolic blood pressure (SBP) were measured one week after the initiation of the experimental diet. Food intake is presented as the mean weight of food eaten per day during the first week of feeding. The *P* values of a one-way ANOVA are shown. *n* = 8 in each group.

Fig. 5. Effects of resveratrol on stroke onset and lifespan in the SHRSP

The day of stroke onset (A) and the survival rate (B) were determined in rats fed the experimental diets containing various doses of resveratrol (0 - 0.05% (w/w)), as described in the Methods section. Control, open circles; 0.001% resveratrol, closed circles; 0.005% resveratrol, open triangles; 0.01% resveratrol, closed triangles; 0.05% resveratrol, open squares. *n* = 8 in each group.

inflammation in this study; however, this effect may be weaker than that of DR, which significantly decreases all levels of IL-1 β , MCP-1 and ICAM-1 in the brain¹³.

Resveratrol Failed to Delay the Onset of Stroke in SHRSP

The SHRSP were fed a control diet or diets containing various doses (0.001 - 0.05%) of resveratrol in order to examine whether resveratrol is capable of delaying the onset of stroke in SHRSP. One week after the initiation of the experimental diets (before which no rats showed any symptoms of stroke), the body weights were slightly lower at all doses in the resveratrol group than in the control group, even though the

food intake was almost the same (Table 3). In addition, no significant differences were observed in systolic blood pressure among the groups. Under these conditions, we monitored the effects of resveratrol on stroke onset and lifespan. As a result, no doses of resveratrol delayed stroke onset (Fig. 5A) or extended the lifespan (Fig. 5B) in the SHRSP. A histological analysis showed that resveratrol decreased the incidence of infarction and edema, but not the incidence or severity of hemorrhage (Table 4). These results indicate that resveratrol slightly suppressed systemic inflammation and the incidence of infarction and edema in the present study, although the effect was insufficient to delay the onset of stroke, especially that involving hemorrhage, in the SHRSP.

Table 4. Magnitude and location of infarction, hemorrhage or edema in individual rats in each group from Fig. 5

Location	Infarction				Hemorrhage				Edema			
	1	2	3	4	1	2	3	4	1	2	3	4
<u>Control</u>												
Rat # 1	–	++	–	+++ ^a	–	++	+	+	++	++	+	+++ ^a
2	–	–	+++	–	–	–	++	++ ^a	–	–	+++	+
3	+	–	–	+	++	+++	+	+	–	+	–	+
4	–	+	+	–	+	+	++	–	+	+	–	–
5	–	–	–	–	–	+	–	–	–	–	–	–
6	–	+++	–	–	+	+++	–	–	+++	+	–	–
7	–	–	–	–	–	+++	+++	–	–	–	–	–
8	–	–	–	–	+	–	–	+	–	–	–	–
<u>0.001% Res</u>												
Rat # 1	–	++	–	–	–	–	–	+	–	++	+	–
2	–	++++	–	–	–	+	–	–	–	–	–	++
3	–	+++	–	–	–	+++	–	–	+	+	–	–
4	–	–	+++	++	–	–	–	–	+++++ ^a	+++++ ^a	+++++ ^a	+++++ ^a
5	++	+++++	–	–	++++	+++++	–	–	–	–	–	–
6	+	++	–	–	+	++	–	–	–	++	–	–
7	–	++++	–	–	+	+++	+	+	–	+++	–	++
8	–	++	++	–	–	++	–	–	–	–	–	–
<u>0.005% Res</u>												
Rat # 1	–	–	–	–	–	+++	–	–	–	+++	+	–
2	–	++	+	++	+++	++	+	++	++++	+	–	+
3	–	–	–	–	–	++	–	++	–	–	–	+++
4	–	–	–	–	–	–	–	–	–	–	–	–
5	–	++	–	–	–	–	–	+	–	–	–	–
6	–	–	+	++	–	–	–	–	–	+	+	+
7	–	–	–	–	–	–	++ ^a	–	–	–	–	–
8	–	++	+	–	–	–	–	+	–	–	–	–
<u>0.01% Res</u>												
Rat # 1	–	–	–	–	+	++++ ^a	++	+	–	–	–	–
2	–	–	–	–	++	+	–	–	–	–	–	–
3	–	–	–	–	+++	–	–	++	–	–	–	–
4	–	–	–	–	–	–	++ ^a	–	–	–	–	–
5	–	–	–	–	+	+++	+	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–
7	+	–	–	–	+	–	+	–	–	–	–	–
8	–	–	++++	–	–	–	++++	–	–	–	++	–
<u>0.05% Res</u>												
Rat # 1	–	–	–	–	–	–	–	–	–	–	–	–
2	–	++	–	–	–	+	–	–	–	–	–	–
3	–	–	–	–	+	–	–	–	+	–	–	–
4	–	–	+++	–	++	–	+++	++++ ^a	–	–	–	–
5	–	–	–	–	+	++	+	+ ^a	–	–	–	–
6	–	–	–	–	+	++ ^a	+ ^a	++ ^a	–	–	–	–
7	–	–	–	–	–	+	–	–	–	–	–	–
8	–	–	–	–	–	–	–	–	–	–	–	–

The brain was removed after death and fixed in 10% buffered formalin solution. 1-4 indicates the location of stroke: 1 is frontal, 4 is in the rear and 2 and 3 are between the frontal region. An increasing number of + indicates a stronger change, – indicates no abnormalities. ^aWhen there were two lesions in the same location, the more severe score is indicated. The rats were numbered in order of the onset of stroke; #1 is the first rat in which stroke occurred in each group.

Discussion

In the current study, resveratrol suppressed the inflammatory gene expression and activated the AMPK signaling pathway in SHRSP CVEC*s in vitro*. In addition, resveratrol partially suppressed systemic and local inflammation in SHRSP *in vivo*. However, resveratrol was unable to delay stroke onset in the SHRSP, although DR markedly delays the onset of stroke and extends the lifespan in SHRSP models¹³.

One reason why resveratrol did not affect stroke onset is that the dose of resveratrol was not sufficient. The plasma levels of resveratrol were about 10 - 60 ng/mL (approx. 0.05 - 0.3 μ M) in the SHRSP fed a diet containing resveratrol. At this concentration, resveratrol partially suppressed systemic and local inflammation *in vivo*, although these effects were weaker than those induced by DR. However, the SHRSP was fed a diet containing 0.001 - 0.05% (w/w) resveratrol in the present experiment, which corresponds to a dose of 10 to 500 mg in humans (60 kg BW) according to the formula for dose translation based on body surface area¹⁸. This dose is usually used in clinical studies and has been shown to have several beneficial effects in human subjects.

Inflammation is recognized to be a key event in stroke onset^{19, 20}, and SHRSP have been shown to exhibit increased systemic and local inflammation prior to stroke. A previous study reported that excess salt loading for two weeks increases the number of ED-1-positive cells (macrophages/activated microglia) in the brain before stroke onset²¹. In addition, inflammatory marker proteins, such as transferrin, hemo-pexin and albumin in the urine and thiostatin in the serum, are detected in salt-loaded SHRSP before the development of stroke²², and acute-phase proteins have also been detected in the cerebrospinal fluid prior to MRI evidence of brain abnormalities²³. Furthermore, the exogenous administration of IL-1 β has been shown to stimulate stroke onset in SHRSP¹². In the present study, resveratrol increased the IL-6 and TNF- α mRNA expression levels in the brain (data not shown). IL-6 and TNF- α have been reported to play a dual role in inflammation and brain damage²⁴⁻²⁶. At this time, it is not clear whether increased IL-6 and TNF- α mRNA expression levels are the result of a protective effect of resveratrol. However, resveratrol did not delay the onset of stroke in SHRSP in this study, although it exhibited weak anti-inflammatory properties *in vitro* and *in vivo*. The beneficial effects of resveratrol have been previously reported in SHRSP models; however, none of these studies examined stroke onset⁹⁻¹¹. The findings of these studies, as well

as the present results, suggest that resveratrol has some beneficial effects *in vivo*, even though the dose was insufficient to delay stroke onset.

In contrast to our results, resveratrol is regarded as being a candidate for disease therapies against obesity, diabetes²⁷, atherosclerosis^{28, 29}, Alzheimer's disease³⁰ and various cancers³¹. Many previous studies have also demonstrated that resveratrol prevents these diseases in animal models, and several clinical studies have been conducted. Resveratrol improves insulin sensitivity and postprandial plasma glucose in subjects with impaired glucose tolerance³². Moreover, resveratrol significantly improves the mean hemoglobin A1c levels, systolic blood pressure and total cholesterol levels in type 2 diabetes mellitus patients^{33, 34}, and treatment with a resveratrol-enriched grape extract was demonstrated to significantly decrease the LDL-cholesterol, apoB and oxidized LDL levels and increase the non-HDL-cholesterol/apoB ratio³⁵. Finally, resveratrol-enriched grape extract treatment also significantly decreases the high-sensitivity C-reactive protein, TNF- α , plasminogen activator inhibitor type 1 levels, as well as the IL-6/IL-10 ratio, and increases the IL-10 level³⁶, and resveratrol therapy has been shown to lead to dose-dependent increases in the cerebral blood flow, although it does not affect the cognitive function³⁷, in addition to reducing tumor cell proliferation by 5% in patients with colorectal cancer³⁸. These findings suggest that resveratrol can prevent various diseases in human subjects. However, it has also been reported that resveratrol does not change the levels of any metabolic or inflammatory markers, oxidative stress indicators or the visceral fat content in obese subjects³⁹. Therefore, further clinical studies are needed to confirm the beneficial roles of resveratrol in preventing stroke in human subjects.

Conclusion

We herein demonstrated that a high concentration of resveratrol affects the inflammatory gene expression in CVEC*s in vitro*. In addition, resveratrol partially suppressed systemic and local inflammation in SHRSP *in vivo*; however, this effect was insufficient to delay stroke onset in the SHRSP, whereas DR markedly delays the onset of stroke and extends the lifespan in SHRSP models. These results indicate that resveratrol does not mimic the beneficial effects of DR against stroke onset. A further clinical study is needed to determine whether treatment with resveratrol is able to suppress the development of stroke in humans.

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

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